Role of Brain Glycogen as a Neuroprotective Agent Against Motor Memory Loss During Times of Hypoglycemia

An Honors Thesis (Honr 499)

by

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Abstract:

Approximately 30 million people are affected by diabetes in the United States alone. Insulin used to treat diabetics can lead to a variety of deleterious effects, including memory loss, during episodes of low blood glucose, or hypoglycemia. The branched polymer of glucose, known as glycogen, is a proposed neuroprotectant against the neurodegenerate affects of hypoglycemia, possibly serving as an alternate energy source to neurons during times of low blood glucose levels. Due to neurons inability to store glycogen as a glucose reservoir, glycogen within astrocytes undergoes glycogenolosis to form the transportable substrate lactate, which will then be shuttled to the neurons. Lactate is then converted back to pyruvate within the neuron and undergoes the TCA cycle, generating energy for the neuron. To test the hypothesis of glycogen acting as a neuroprotectant during times of hypoglycemia, mice with and without brain glycogen were exercised using a motor memory instrument, rotarod, while in various glycemic states. Brain tissue was then harvested and subjected to a western blot in order to analyze the expression of memory proteins. When compared to wildtype, mice lacking brain glycogen have impaired memory formation during periods of hypoglycemia. Therefore, it is expected that mice lacking brain glycogen will have lower expression of memory proteins. Mice lacking brain glycogen also have higher levels of lactate within the blood following rotarod exercise, suggesting lactate's role as a compensatory mechanism for memory formation when blood glucose is unavailable.

Acknowledgments

I would like to thank Dr. Bartholomew Pederson for his continued patience and assistance in this thesis project. He has served as an upstanding mentor and helping hand through my years in research.

I would like to thank my lab partners Dan, Cody, Hannah, Cale, Brittany, Logan as well as the lab technician Justin Crowder for their continued support of the project. I would also like to that Staci Weaver for all the rotarod work she completed while in the lab.

Process Analysis Essay:

This thesis project took place over a span of 6 semesters, beginning in the spring of my sophomore year. The prevalence of the research to diabetes and its potential to assist in identifying an effective therapy to treat diabetic patients that doesn't expose them to cognitive impairment associated recurrent hypoglycemia motivated me throughout the project. The brains main source of energy is glucose, thus a polymer of glucose, known as glycogen, potentially serves as an emergency energy store when glucose is not readily available to the brain during times of hypoglycemia. Glycogen metabolism and its potential role as a neuroprotectant against hypoglycemic memory loss is the central focus of this thesis and other experimental work conducted in this lab.

Two mouse models were utilized in this lab, one null of any brain glycogen and one with normal levels of brain glycogen, in order to determine the role glycogen plays in memory formation. Based on previous reports that glycogen plays a role in memory formation during times of hypoglycemia, we hypothesized that mice lacking brain glycogen would have impaired memory formation when compared to WT mice. In order to test this hypothesis, mice were subjected to a single treadmill rotarod machine over a course of four trials. During trials 2 and 3 hypoglycemia was induced using insulin in order to evaluate memory formation during insulin-induced hypoglycemia. The results of this experiment supported our hypothesis, indicating that glycogen does in fact play a neuroprotective role in memory formation during times of hypoglycemia.

The thesis project required hours of work to be set-aside during the week to use the rotarod machine and conduct bench work, as well as years of study to reach an appropriate sample size. Even prior to the experimental procedures, hours of reading

were required in order to fully understand glycogen metabolism and its possible role in diabetes therapy. At times the experimental procedure would be compromised or experimental errors would occur while performing bench work, thus compelling me to preserve through the tough times in order to reach my final goal. The project instilled in me a patience and appreciation for research previously absent. The entirety of the experiment consisted of increased levels of stress, but adjusting to this stress not only assisted me in the lab but also helped me preserve through the stress associated with things outside of the lab such as academia. The patience I accumulated while working in the lab helped me take challenges such as dental school applications/interviews or final exams head on. I found I was better able to deal with problems in everyday life due to the values instilled in me while in the lab.

Aside from patience and perseverance, the thesis project required a lot of teamwork and coordination with other members of the lab. The teamwork and leadership skills I inherited throughout my time working on my thesis are essential to the success I am able to obtain outside of the lab. All in all, the entire project not only resulted in a final thesis, but in the growth of my personal character. It is my hope and belief that the results from my thesis project will add to the diabetic research communities knowledge on the role of glycogen metabolism during times of hypoglycemia, eventually leading to an alternative therapy to insulin treatment. On a smaller scale, the hard work I put into this lab will motivate future undergraduate students to take on a role in research, whatever the field may be. In Dr. Pederson's lab in particular, it is my hope that my leadership skills assisted many of the younger students in lab protocol and etiquette.

Introduction

Diabetes mellitus, characterized by recurrent exposure to hyperglycemia consequential of insulin secretion defects or insulin resistance (2006), affects approximately 30 million Americans and is currently the 7th leading cause of death in the U.S (2012). If left untreated, diabetes results in long-term exposure to hyperglycemia leading to organ failure in a variety of vital organs, including the kidneys and eyes, and impaired cognitive function (Reijmer 2010). Diabetes mellitus can manifest in one of two forms, type I or type II diabetes. Type I diabetes, most commonly diagnosed in children, is characterized by a complete deficiency of insulin production by pancreatic beta cells. Type II diabetes, the more prevalent of the two forms, is caused by a resistance to the insulin naturally produced in the body. Normal blood glucose levels, known as euglycemia, occur at approximately 4.5 mmol/L (Reijmer 2010), however, the hyperglycemia consequential of diabetes results in blood glucose levels at or above 11 mmol/L. Insulin therapy is a common treatment method utilized to return blood glucose to normal levels (Herzog 2011).

Insulin administration often times results in abnormally low blood glucose levels, known as hypoglycemia, and is thus the principle obstruction to maintaining normal glucose metabolism and blood glucose levels (Amaral 2012). Glucose is the central fuel source to the brain, thus brain activity is extremely reliant upon cerebral glucose metabolism (Amaral 2012). When blood glucose drops to hypoglycemic levels, 2.0mmol/L, glucose in the brain is almost entirely depleted due to the elevated glucose metabolic rates needed to produce the energy necessary to support neuronal function (Herzog 2011). Low brain glucose levels have been shown to have a neurodegenerate

effect leading to impaired cognitive function and memory formation (Suzuki 2011). On average, diabetic patients utilizing insulin therapy to treat their hyperglycemia, experience mild hypoglycemia about twice a week (Bolo). Severe hypoglycemia can lead to seizures, neuronal cell death, and comas if consistently induced. Due to the prevalence of hypoglycemia and the resulting cognitive impairment, identifying alternative therapies that affectively limit exposure to hyperglycemia without inducing hypoglycemia is of paramount concern (Herzog 2011).

Glycogen, the branched polysaccharide utilized by humans to store and utilize the brains main energy source glucose (Figure 1), has been proposed to play a possible neuroprotective role during times of hypoglycemia. Glycogen synthesis, glycogenesis, results from the enzyme glycogen synthase generating alpha 1-4 bondages between glucose monomers and only occurs in astrocytes (Suzuki 2011). Neurons inability to synthesize and store glycogen indicates an essential supportive role of astrocytes in neuronal cell function (Suzuki 2011). Stored glycogen within the astrocytes can be degraded by glycogen phosphorylase, eventually yielding lactate that can be shuttled across monocarboxylate transporters (MCTs) into neurons where it can be utilized to generate the energy necessary for neuronal function (figure 2) (Suzuki 2011). It has been shown that this astrocytic glycogen metabolization is required for long-term memory formation and neuronal cell functions (Suzuki 2011). Ana Amaral proposed in her hypoglycemia review that the interconnected neuronal-astrocyte metabolism results in a redox coupling reaction that allows astrocytes and neurons to differentially select either lactate or glucose to use as an energy source, implying lactate and other glycogenolysis substrates act as alternative energy sources capable of rescuing memory formation during

times of hypoglycemia. The proposed neuroprotective role of glycogen makes it the central focus of this lab and the experiments completed within this lab.

Of particular interest is the effect hypoglycemia has on formation of motor memory, or the process of encoding a complex movement sequences within the brain (Chagniel 2013). Expression of motor memory proteins such as Creb, pCreb, and Arc has been shown to increase following exposure to motor memory tests (Chagniel 2013 and Min 2014), therefore analyzing protein expression in brains of mice containing differing levels of brain glycogen could help reveal whether glycogen plays a neuroprotective role. For this project, motor protein KIF17 was designated to evaluate memory formation. To our knowledge, KIF17 has yet to be directly correlated to motor memory formation, however it has been shown that the protein is interconnected to Creb and pCreb phosphorylation cascades (Roberson 2008). Mice were trained for motor skill of running using a single treadmill rotarod machine in order to measure motor memory formation. Motor learning occurs in both fast and slow learning stages within the cerebellum and hippocampus of the brain (Doyon 2009). Accordingly, mice were subjected to the rotarod treadmill at two differing intensities, the first being an accelerating speed from 3-30 RPM and the second a consistent speed of 28RPM. To interpret whether or not KIF17 expression is directly affected by motor memory formation, different regions of the brain (hippocampus, cerebellum, and cortex) were harvested following rotarod exposure and subjected to a western blot analysis.

We hypothesized that glycogen plays a neuroprotective role in motor memory formation during times of hypoglycemia, thus mice containing brain glycogen would have increased latency on the rotarod and show increased expression of memory proteins.

Genetically altered mice null for brain glycogen were compared to WT mice in order to examine glycogen's neuroprotective role. The single treadmill rotarod was utilized to test the formation of motor memory. Mice were subjected to four trials over a course of eight days and hypoglycemia was induced during trials 2 and 3 to evaluate the cognitive effects of low brain glucose. On the last trial of rotarod, WT mice previously treated with insulin were able to sustain balance on the rotarod for a time similar to those treated with saline, where as insulin treated mice lacking brain glycogen experienced significantly shorter latency on the treadmill machine. To further support glycogens neuroprotective role, percent improvement across the four trials was calculated and once again WT mice had significantly higher improvement rates than mice lacking brain glycogen, suggesting glycogen assisted in memory formation during times of hypoglycemia. Western blotting of hippocampal and cerebellum brain tissue indicated expression of KIF17 at 170kD, following exposure to the rotarod machine. However, the western provided no conclusive evidence as to whether or not WT mice had higher KIF17 expression levels than mice lacking brain glycogen.

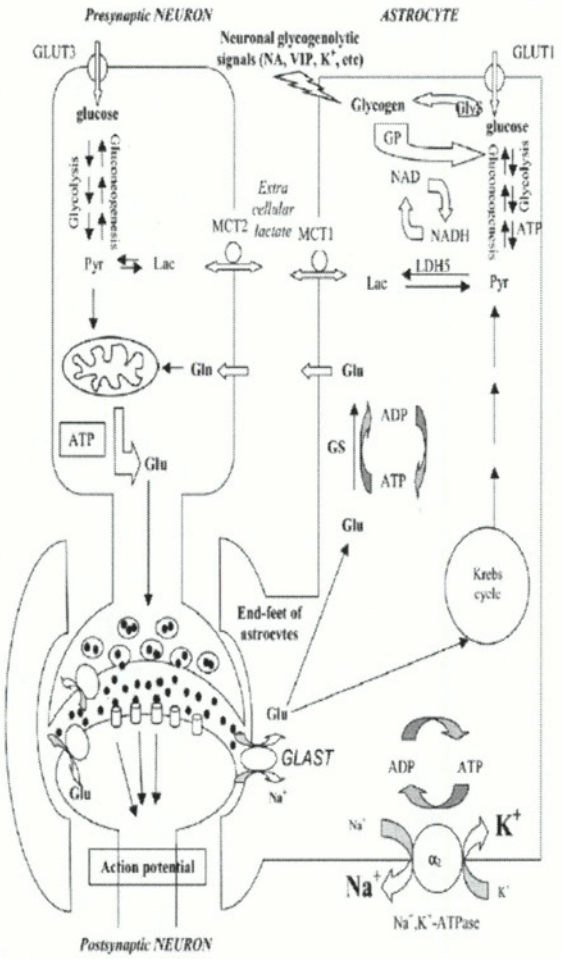

Figure 1: Utilization of glucose as energy source once entering brain cells. Glucose enters neurons and astrocytes through GLUT3 and GLUT1 transporters respectively and then enters glycolysis, generating both ATP and pyruvate. In order to generate ATP pyruvate undergoes the TCA cycle, followed by oxidative phosphorylation.

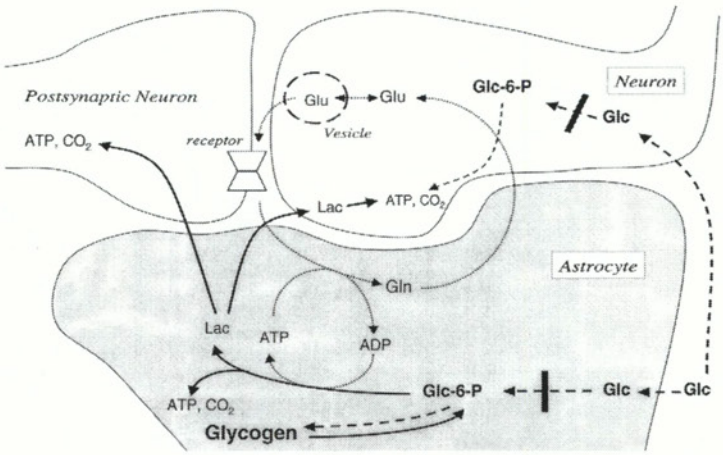

Figure 2: Glycogen metabolism in the astrocytes supplies neurons with an alternate energy source when blood glucose levels are low. In astrocytes, glucose entering the brain can be used to generate ATP or stored as glycogen. When blood glucose levels are low, glycogen can be metabolized into lactate which can be shuttled from astrocytes into neurons where it can be converted back into pyruvate to generate ATP.

MATERIALS AND METHODS

Generation of MGSKO/GSL30 mouse line

Lexicon Genetics Incorporated provided mice heterozygous for the glycogen synthase gene *GYS1* (Pederson 2004). Embryonic stem (ES) cells from the Lexicon Genetics Omnibank library of gene trapped ES cells were infected with VICTR25, a retroviral gene trap vector, upstream of exon 12 on the *GYS1* gene (Pederson 2004). Embryonic stem cells containing a normal karyotype of the disrupted *GYS1* gene were then injected into C57BL/6J blastocysts and these blastocysts implanted into pseudopregnant female mice. Pups resulting from the C57BL/6J blastocyst implantation were crossed with C57BL/6J X 129/SvJ mice and gene disruption was confirmed by Southern Analysis (Pederson 2004). These mice were then mated to generate a mouse model null of the *GYS1* gene, referred to as muscle glycogen synthase knockout

(MGSKO). Pups homozygous for *GYS1* disruption ($GYS1^{-/-}$) were affected by both venous and pulmonary congestion, resulting in just a 10% survival rate (Pederson 2004).

Due to low survival rate, a more viable mouse model null for glycogen synthase in the central nervous system was needed. To accomplish this, MGSKO mice were crossed with mice expressing increased levels of the glycogen synthase protein in the muscle, denoted as GSL30 (Manchester 1996). GSL30 mice contain a creatine kinase gene promoter that directs muscle specific expression of *GYS1,* causing an overexpression of glycogen synthase in the skeletal muscles, but no increase in the brain (Manchester 1996). These GSL30 mice were crossed with $GYS1^{-/-}$ mice, resulting in $GYS^{+/-}$ mice with transgene expression (Pederson 2004). $GYS1^{+/-}$ mice with transgene expression were mated with other $GYS1^{+/-}$ mice in order to generate mice null of any endogenous glycogen synthase, but overexpressed glycogen synthase in the muscle, $GYS1^{-/-/tg}$. The result of this cross was a viable mouse line containing no glycogen in the central nervous system (MGSKO/GSL30). A multiplex as well as a uniplex PCR reaction was used to confirm the genotype of the MGSKO/GSL30 line, using toe clippings from each experimental mouse.

Mouse Husbandry

Mice were kept in a temperature and humidity controlled room with a 12-hour light and dark cycle. Mice had access to food and water at all times unless stated otherwise in procedures requiring the mice to be fasted over night. The Ball State University Animal Care and Use Committee approved all procedures and husbandry.

Experimental mice

Male mice of approximately three months of age, from the MGSKO/GSL30 line were used during this experiment. Genotypes *GYS1*$^{+/+}$ (WT) and *GYS1*$^{-/-/tg}$ were used to test glycogen's proposed ability to act as a neuroprotective agent.

MOTOR MEMORY EXPERIMENTAL PROCEDURES

Rotarod – fed state motor learning

Before subjecting the mice to rotarod during times of hypoglycemia, mice were subjected to the rotarod (figure 3) while in the fed state, acting as positive control. The mice were subjected to four experimental trials on the rotarod over eight separate days. Latency, the amount of time spent on the rotarod before falling, was measured during each trial. Latency measurement was concluded in one of three ways: the mouse rotated in a full circle around the rotarod, the mouse fell from the rotarod, or the mouse reached the maximum allotted time limit. An increase in latency over the four trials would indicate motor learning is occurring over the course of the eight days.

Mice were subjected to four trials consisting of two differing speed settings during each experimental day. During trial 1 (Day 1), the mouse was placed on the rotarod subjected to an initial speed of 3.0 RPM that would accelerate to as many as 30 RPM. The amount of time spent on the rotarod was measured up to a maximum of five minutes. The mouse was then taken off the rotarod and allowed to rest for 30 seconds before being placed back on the rotarod and subjected to a constant speed of 28 RPM for

a maximum of 90 seconds. This condition was repeated twice more with 30 sec of rest between each, and latency was measured each time. In between individual mice, the rotarod was wiped down using 66% ethanol in order to eliminate any odor from previous mice that may affect future mice's performance. The same protocol was used during trial 2 (day 2), trial 3 (day 6), and trial 4 (day 8).

Rotarod – hypoglycemic motor learning

Mice were subjected to either saline or insulin injections prior to rotarod in order to assess glycogen's neuroprotective role in motor learning during times of hypoglycemia. This motor memory test took place using a single rotation rotarod treadmill (Med Associates Inc ENV-567M) and mice were subjected to four trials occurring over a course of eight separate days. During each trial final latency measurement was concluded as described above.

For trial 1 (day 1) mice were moved into the experimental room containing the rotarod 30 minutes prior to exposure to the rotarod (~1:30pm). Four mice, two *GYS1* $^{+/+}$ and two *GYS1* $^{-/-/tg}$ were brought into the room to undergo the rotarod experiment. One mouse would be exposed to the rotarod at a time and ~15 minutes was placed between the end of one mouse's attempts and the beginning of the other's. Blood glucose was measured from tail vein, as described below, prior to the rotarod experiment and the tail was cauterized to prevent further bleeding. Each mouse underwent 4 attempts on the rotarod with ~30 seconds between attempts. During attempt one the rotarod rotates at an accelerating speed from 3.0RPM to 30RPM for a maximum of five minutes. Following

the first attempt, the mouse would then undergo three attempts on the rotarod spinning at a constant speed of 28 RPM for a maximum of 90 seconds.

The night prior to trials 2 (day 2) and 3 (day 6), mice were fasted at ~5pm. On the day of the experimental trial mice were moved into the experimental room half an hour prior to injections (~12:30pm). Each mouse was weighed to the nearest 0.1g and a syringe would be prepared for each mouse containing either saline or insulin. One WT mouse would receive insulin, while the other would receive saline and the same parameters were in place for *GYS1* $^{-/-/tg}$ mice. The first mouse had its blood glucose measured, followed by an intraperitoneal injection of the appropriate dose, as indicated below, at ~1pm. Fifteen minutes was left between each injection, thus mouse 1 received an injection at 1pm, mouse 2 at 1:15pm, mouse 3 at 1:30pm, and mouse 4 at 1:45pm. Following the initial injection blood glucose was measured 30 minutes following the injection, 60 minutes following the injection, and immediately after the mouse completed the final attempt on the rotarod. Sixty minutes after injection, mice were then exposed to the rotarod under the same conditions as used in day 1 (a single attempt at an accelerating speed of 3.0RPM to 30RPM, followed by three attempts at a constant speed of 28RPM).

For trial 4 (day 8) mice were moved into the experimental room half an hour prior to the experiment (~1:30pm) and the same experimental procedure used during trial 1 (day 1) was followed.

Hypoglycemia induction

The night prior to rotarod experiments requiring induced hypoglycemia, mice were fasted starting at 5pm. To ensure the mice had no access to food, the mice were

placed in a new cage with fresh bedding and the food container was emptied. The following day insulin preparation began at 12:30, approximately half an hour before the first injection. Each mouse used for the experiment was placed in a balance and measured to the nearest 0.1g. Insulin, 70/30 Humulin Insulin (Eli Lilly), was diluted with 0.9% saline such that that administration of 5µl/g body weight resulted in a dose of 0.6U/kg for WT mice and 1.0 U/kg for *GYS1* $^{-/-/tg}$ mice. Saline (0.9%) was administered at 5µl/g body weight for all mice, regardless of genotype, not undergoing hypoglycemia. Mice were administered either insulin or saline intraperitoenally, based on treatment group, one hour before they were subjected to the rotarod attempts. Following rotarod attempts the mice were put back into their home cage and re-fed.

Blood glucose measurements

Blood glucose was monitored in each mouse prior to injection, 30 minutes following the injection, 60 minutes following the injection, and following the final rotarod attmpt. Blood glucose was monitored using HemoCue Glucose 201 microcuvettes and a HemoCue Glucose 201 glucometer. Each mouse had a small portion of its tailed clipped using dissection scissors. The tail was then milked to extract approximately 5µl of blood that would then be loaded into a HemoCue Glucose 201 microcuvette. This microcuvette was then wiped with a kimwipe (KIMTECH) to remove any excess blood and placed in the glucometer to be read.

Lactate measurements

Lactate levels in the blood were measured prior to the rotarod trial (60 minutes after injection) and following the rotarod trial. In order to measure lactate, LactatePlus strips (Nova Biomedical 40813) were inserted into a LactatePlus lactate reader (Nova Biomedical 40828). Similar to glucose readings, the tail was milked for blood and this blood was loaded into the lactate strips and placed into LactatePlus reader. Following the reading, a silver nitrate stick was used to cauterize the tip of the tail and prevent further bleeding.

MOTOR MEMORY PROTEIN ANALYSIS

Hippocampus removal

In order to investigate the expression of proteins involved in motor memory formation, the brain from each experimental mouse was harvested approximately 24 hours following the final rotarod trial. To harvest the brains, mice were decapitated and the head placed in diluted 1x phosphate buffered saline (PBS) that is formulated to mimic physiological serum conditions. The brain was then dissected using dissection scissors and blunted forceps preventing damage to the tissue. Once removed, different regions of the brain (hippocampus, cerebellum, cortex) were dissected in order to evaluate motor memory protein expression in differing areas of the brain. To dissect the cerebellum, a scalpel was used to detach the cerebellum from the cortex of the brain. Next the cortex was cut in half using a scalpel and the tissue covering the hippocampus was peeled back and the hippocampus was removed using a blunted surgical spatula (figure 4). Each region of the brain was placed into a separate liquid nitrogen-filled scintillation tube

following dissection. Brain tissue was then powdered using a tissue pulverizer and stored at -80°C.

Tissue Homogenation

In order to evaluate motor memory protein expression in the brain, tissue was homogenized to lyse and isolate the proteins from the cell membrane. Brain tissue was homogenized utilizing a method described by Roberson *et al.* The isolated protein was quantified using the Bradford assay to determine protein concentration in each sample for ensuing western blot analysis.

Brain samples containing hippocampus, cortex, and cerebellum brain tissue were moved from -80°C freezer and immediately placed into liquid nitrogen. Tissue from each sample was weighed (40-50mg) and relocated to nitrogen-cooled microfuge tubes. Samples were then thawed on ice for 1 minute and homogenized on ice for 30 seconds, in 10 volumes (ie.400µl/40 mg) of homogenization buffer (0.2M NaCl, 0.05M Tris, 2mMNaF, 2.5mM EDTA (sigma E5134), DTT (1M), Na_2,P_2O_7, protease inhibitor (sigma P8340),100µM sodium orthovanadate) using a tissue tearor at its maximum speed setting (Biospec Products, 985370-04) After all samples were homogenized, tubes were centrifuged for 15 minutes at 16,000xg at 4°C. Once centrifuged, 50µl of each sample was aliquoted to fresh centrifuge tubes and stored at 4°C for future Bradford assays. Another 200µl of supernatant from each centrifuged sample was placed in a separate, fresh centrifuge tube, mixed with 5X SDS-PAGE loading buffer with BME (beta mercaptoethanol Sigma M7522) and placed on ice. Samples were then heated at 70°C for 10 minutes and then stored at -20°C for subsequent western blotting.

Bradford assay

Following homogenization, a Bradford assay was preformed to determine protein concentrations of samples. Based on protein concentrations, appropriate amounts of protein were calculated to ensure equal protein loading for Western blotting.

Samples set aside for a Bradford assay during tissue homogenization were taken from 4°C storage and maintained on ice. BSA (2mg/ml, ThermoScientific PA196779) solutions of differing dilutions (0%, 0.05%, 0.1%, 0.25%, 0.5%) were prepared in order to compare protein concentrations of samples to a standard curve. BSA solutions and experimental samples, at a 1:10 dilution of sample to water, were loaded into 96 well plate and mixed with Thermo Coomassie Bradford reagent (ThermoScientific 1856209). The plate was then mixed on a microplate genie and read on a microplate reader (EL_x800 UV BioTek Instruments) to quantify protein concentration using spectrophotometry.

Western Blotting

Samples set aside for western blot analysis were removed from -20°C storage and set on ice to thaw. While samples were thawing, agarose SDS-PAGE gels were prepared in two steps. First a 10% separating gel was constructed (acrylamide (BioRad 37 5:1), 1.5M Tris-Cl pH 8.8 with SDS, ELIX water, ammonium persulfate, TEMED (BioChemika 87689)) and poured between two electrophoresis plates. The stacking gel sat for 45 minutes at room temperature to polymerize. Once polymerized, 5% stacking gel (acrylamide (BioRad 37 5:1), 0.5M Tris-Cl pH 6.8 with SDS, ELIX water, ammonium persulfate, TEMED (BioChemika 87689)) was poured over the separating

gel, a comb was inserted in the solution creating wells for samples and the gel was allowed to polymerize at room temperature for 45 minutes.

Once the gel was fully polymerized, 1X running buffer (5X Tris/glycine/SDS running buffer and Elix) was poured between the plates and electrophoresis tank. Agarose gel (2% agarose solution) prevented leakage of running buffer between the plates. The initial well in the gel was filled with 5µl of ThermoScientific PageRuler pre-stained molecular weight ladder (ThermoScientific 26616). Remaining wells were then filled with 30µl of brain tissue samples in 1X SDS loading buffer. Electrodes were then hooked up to the electrophoresis apparatus and the samples were run at 200V for one hour. Upon completion the plates of the cassette were gently separated and a transfer sandwich was prepared. The sandwich was layered in the following order: scotch brite pad, 3MM paper, gel, nitrocellulose membrane (Amersham Protran 0.45µm NC), 3MM paper, scotch brite pad. The sandwich was placed into the transfer apparatus (BioRad) and run at 100V for 90 minutes in transfer buffer (Trizma base (Sigma T1503-1KG)/Glycine (Gold Biotechnology G-630-1)/methanol (VWR analytical BDH2018-5GLP)/elix)

Following the transfer, the sandwich was dismantled and the membrane removed. The membrane was then stained in 0.1% Ponceau stain (ponceau (Sigma 094K3651), glacial acetic acid (Sigma SHBC9403V), type 2 water) for one minute in order to preview total protein on the membrane (figure 5). Ponceau stain was removed and the membrane was then rinsed in type 1 water to remove excess stain. Following imaging of the membrane, the membrane was cut in half to blot with antibodies against loading control and experimental protein of interest separately. The membrane was then washed

in 1XTBS/ 0.1% tween (5XTBS (200mM Tris/500mM NaCl pH 7.5), type 2 water, tween (Sigma SLBH5836V) until pink stain on membrane was completely gone.

Following staining, 1XTBS/Tween/3% milk blocking solution was prepared. Membranes were submerged in the blocking solution and blocked with rocking at room temperature for 2 hours. Blocking solution was discarded following the hour block and antibodies were prepared. KIF17 (Sigma K3638) primary antibody was diluted (1:600) in 1XTBS/0.1% Tween/ 3% skim milk (LabScientific M0841) and GAPDH (Sigma NB300) primary antibody was diluted (1:5000 in 1XTBS/ 0.1% Tween/ 2% milk). Membranes containing the protein of interest (KIF17) were submerged in KIF 17 antibody, while membranes containing the loading control protein (GAPDH) were submerged in GAPDH diluted antibody. These membranes were then rocked with the primary antibodies overnight at 4°C.

Secondary Antibodies were prepared the following day for both KIF17 (1:5000 dilution of goat anti-rabbit IgG (Sigma A6154) in1XTBS/0.1%Tween/3% milk and for GAPDH (1:2000 dilution of anti-mouse antibody (Novus HAF007) in 1XTBS/0.1%Tween/3%milk). Secondary antibodies were poured into reagent tubes that were then secured into the appropriate position on the Blotbot (NextAdvance Freedom Rocker BlotBot 240). Primary antibodies were then removed from the membranes and membranes were placed into appropriate trays in BlotBot. The BlotBot was programmed to complete the blot with the following steps:

- Wash in 1XTBS/0.1%Tween two separate times for 10 seconds each
- Wash in 1XTBS/0.1%Tween three separate times for 10 minutes each

- Rock in secondary antibody at room temperature for 1 hour
- Discard secondary antibody solution
- Wash in 1XTBS/0.1%Tween two separate times for 10 seconds each
- Wash in 1XTBS/0.1%Tween three separate times for 10 minutes each

Following blotting in the BlotBot, ECL reagent (Supersignal West Pico-Thermoscientific #34079) was prepared mixing 1.25 mL of supersignal west stable peroxide solution and 1.25mL of supersignal west pico luminol/enhancer solution. Membranes were removed from blotting tray, drained of solution by blotting on kimwipe, and placed protein side up in clean dish. A transfer pipet was then used to add ECL solution directly to the surface of each membrane and allowed to incubate for ~5 minutes. The membrane was then drained of the ECL reagent and blotted to remove remaining liquid. The dried membrane was then wrapped in plastic wrap (Glad ClingWrap) and molecular weight markers on ladder were marked using sharpie marker. Wrapped membranes were placed on Kodak imager (Kodak DigitalScience Image Station 440) to be imaged using Kodak 1D 3.6 software. The membranes were previewed first to ensure "image overlay" between the preview and ECL would be accurate. Once preview of the membrane was captured, the imager was closed and the ECL membrane exposed for an appropriate amount of time. Using Kodak 1D 3.6 software the final images were adjusted using differing contrasts to provide best quality available.

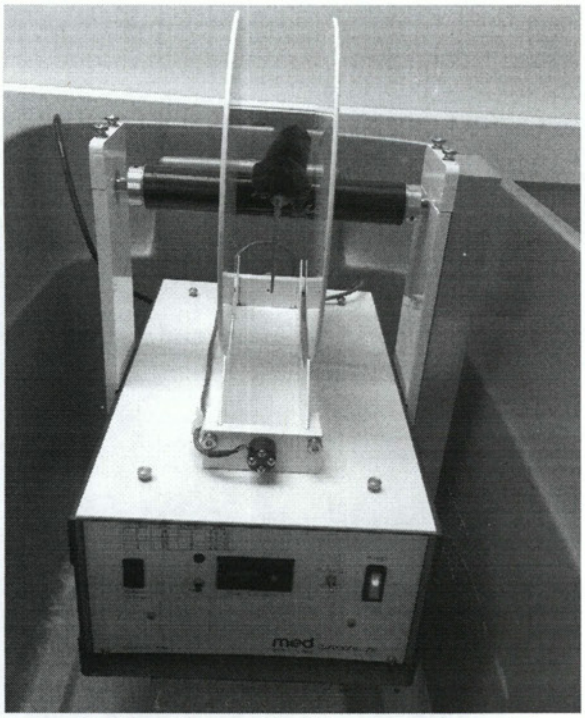

Figure 3: Single treadmill rotarod machine was utilized in order to measure motor memory formation. Mice underwent the rotarod over a series of four trials in order to measure their ability to form memory of new motor skills. The speed of the treadmill was set to either level 7 (3-30RPM) or level 3 (28RPM) in order to expose mice to differing speeds on the rotarod.

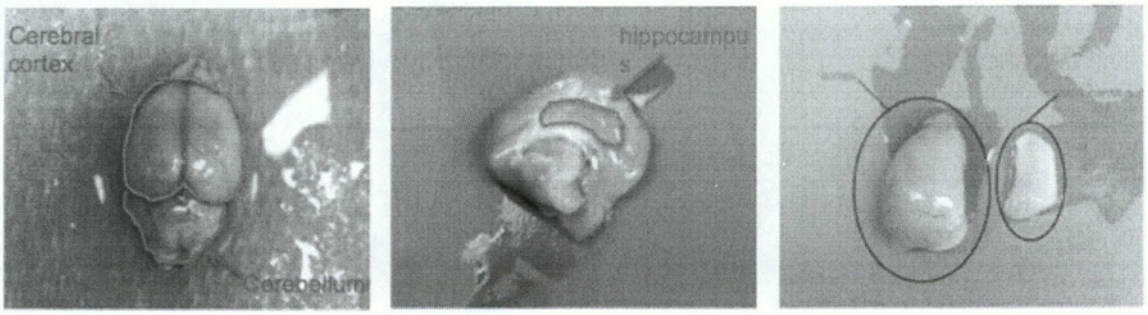

Figure 4: Brain tissue was dissected from mice that previously underwent rotarod in order to track protein expression using a western blot analysis. The brain cortex, cerebellum, and hippocampus were all dissected from the brain and placed in liquid nitrogen to conserve the metabolites within the tissue. The tissue was then powdered, homogenized, and then analyzed using a western blot.

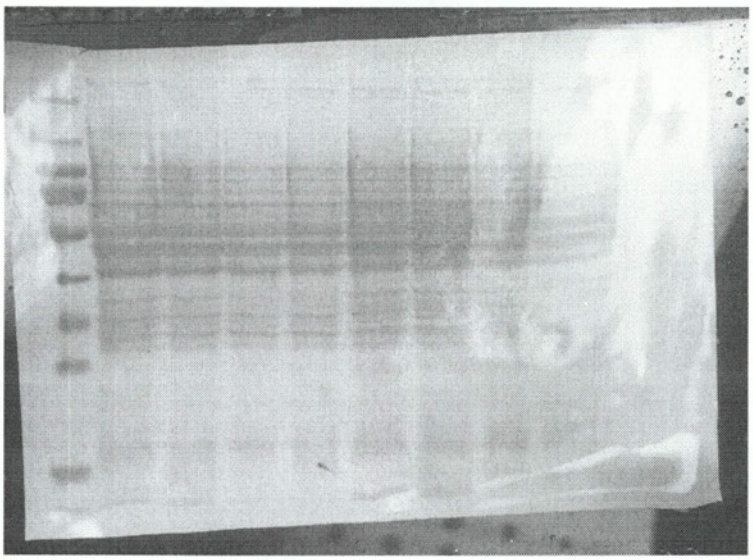

Figure 5: Membranes are stained using ponceau stain to show total protein expression. Following transfer from gel onto a membrane, proteins were stained using a ponceau stain to give a preliminary indication as to whether or not equal concentrations of protein were loaded into each lane.

RESULTS

Rotarod- Fed State Motor Learning

To determine if brain glycogen is important for motor memory formation, a former student in Dr. Pederson's laboratory subjected mice with or without brain glycogen to the single rotation rotarod treadmill in the fed state. Mice were exposed to two alternative speeds while on the rotarod and motor memory was traced for each speed setting using latency measurements, or time to fall. Mice were first subjected to the rotarod with an accelerating speed from 3.0RPM up to 30RPM (level 7). Following one attempt at level 7, mice were then subjected to three consecutive attempts at a constant speed of 28RPM (level 3).

Latency on rotarod in fed state on rotarod set to level 7

Latency was measured in both *GYS1* $^{+/+}$ (WT) and *GYS1* $^{-/-/tg}$ mice over a course of four trials (figure 6). There was no significant difference in latency to fall between WT and *GYS1* $^{-/-/tg}$ for all trials (trials 1-4). Percent improvement in latency from trial 1 to trial 4 while in the fed state was also insignificant between WT and *GYS1* $^{-/-/tg}$.

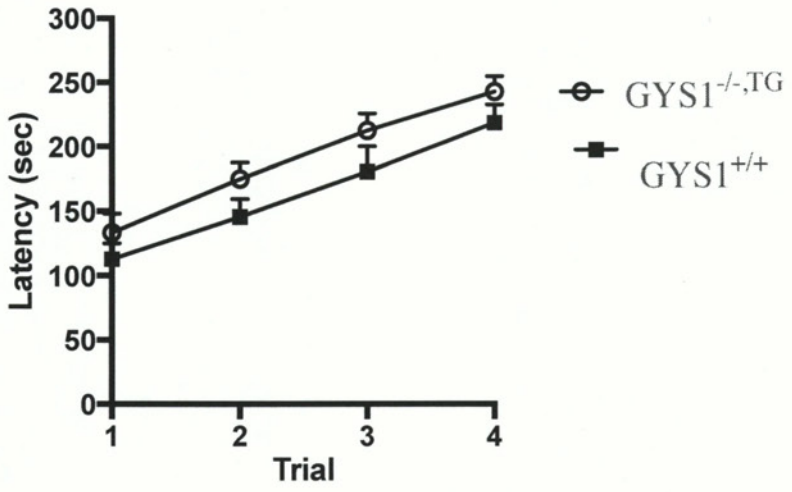

Figure (6): Latency on rotarod set to level 7 increased consistently over four consecutive trials across in both WT and *GYS1* $^{-/-/tg}$ mice in the fed state. Latency was measured in both *GYS1* $^{-/-/tg}$ (n=29) and WT (n=21) mice. Mice were in the fed state through all four trials. There was no difference in percent improvement from trial 1 to trial 4 between WT and *GYS1* $^{-/-/tg}$. Significance was determined using a non-parametric t-test.

Latency on rotarod in fed state on rotarod set to level 3

Latency was measured in both *GYS1* $^{+/+}$ (WT) and *GYS1* $^{-/-/tg}$ mice over a course of four trials (figure 7). There was no significant difference in latency to fall between WT and *GYS1* $^{-/-/tg}$ for trials 1,2, and 4. During trial 3 *GYS1* $^{-/-/tg}$ mice displayed a shorter latency time compared to WT mice ($P<0.0001$). Percent improvement in latency from trial 1 to trial 4 while in the fed state was also insignificant between WT and *GYS1* $^{-/-/tg}$.

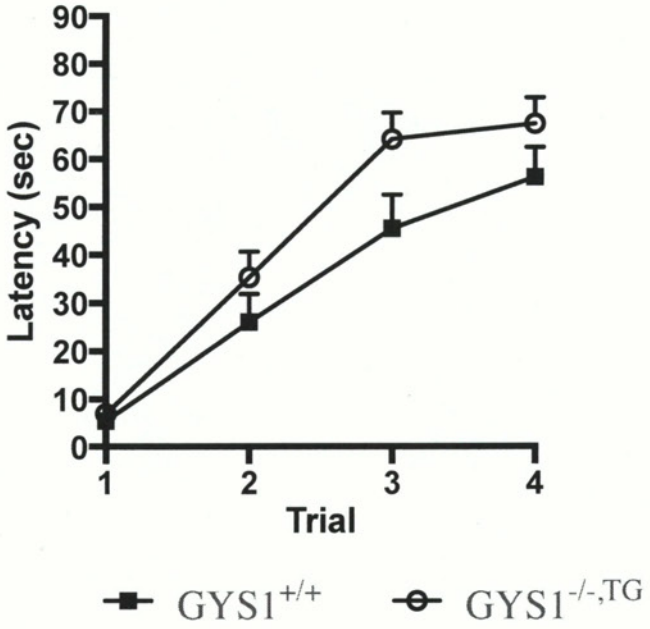

Figure (7): Latency on rotarod set to level 3 increased consistently across genotypes over four consecutive trials in both WT and *GYS1*$^{-/-/tg}$ in the fed state. Latency was measured in both *GYS1*$^{-/-/tg}$ and WT mice. N was between 21-29 for each group. Mice were in the fed state through all four trials. There was no difference in percent improvement from trial 1 to trial 4 between WT and *GYS1*$^{-/-/tg}$. Significance was determined using a non-parametric t-test.

Rotarod- Hypoglycemic Motor Learning

Blood glucose levels

My studies examined the affects of hypoglycemia on memory. To assess the effect of hypoglycemia on motor learning, mice were subjected to four trials on the single rotation rotarod treadmill over a course of eight days. Mice were in the fed state during trials 1 and 4 and in fasted state during trials 2 and 3. During trials 2 and 3 mice were injected with either insulin to induce hypoglycemia or the vehicle saline. Blood glucose

was measured just before and immediately following rotarod treatment during trials 1 and 4. During trials 2 and 3 blood glucose was measured prior to injection, 30 minutes following injection, 60 minutes following injection, and immediately following rotarod.

Blood glucose levels during trial 1 (day 1):

Blood glucose was monitored in both WT and *GYS1* $^{-/-/tg}$ mice prior to and following rotarod assessment. There was no significant difference in blood glucose levels between WT and *GYS1* $^{-/-/tg}$ mice either prior to or following rotarod assessment (Figure 8). Post-rotarod blood glucose levels were significantly higher when compared to pre-rotarod blood glucose levels in both genotypes of mice ($P<0.01$) (figure 8). On average, WT blood glucose levels were 13% higher following rotarod assessment when compared to pre-rotarod blood glucose, while *GYS1* $^{-/-/tg}$ blood glucose levels were 16% higher following rotarod assessment compared to pre-rotarod blood glucose levels.

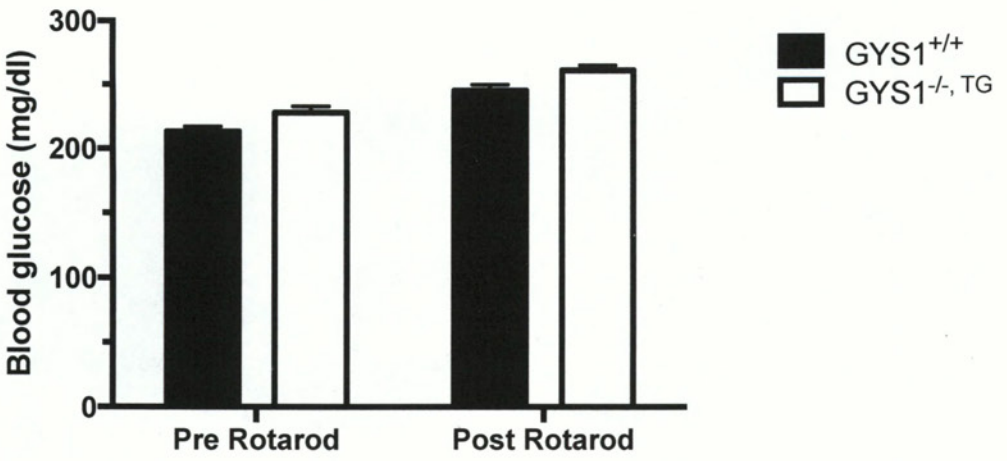

Figure (8): Blood glucose levels increased following rotarod assessment **in both WT and *GYS1* $^{-/-/tg}$ mice.** Blood glucose was measured in both WT and *GYS1* $^{-/-/tg}$ mice prior to and immediately following exposure to the rotarod instrument. N=38-40 for each group. Significance was measured using a Newman-Keuls multiple comparison test within a one-way ANOVA.

Blood glucose levels during trials 2 (day 2) and 3 (day 6):

During trials 2 and 3, blood glucose was monitored in both WT and *GYS1* $^{-/-/tg}$ mice prior to injection, 30 minutes following injection, 60 minutes following injection, and following rotarod assessment. During both trials 2 and 3, both WT insulin treated and *GYS1* $^{-/-/tg}$ insulin treated groups had significantly lower blood glucose levels 30 minutes following injection, 60 minutes following injection, and immediately following rotarod assessment when compared to the vehicle treated groups ($p<0.0001$) (figure 9). Consistent in both trials 2 and 3, there was no significant different in WT and *GYS1* $^{-/-/tg}$ blood glucose levels prior to and following rotarod assessment. On average insulin treated WT mice were at a blood glucose level of 54 mg/dl and insulin treated *GYS1* $^{-/-/tg}$ were at an average blood glucose level of 55 mg/dl (figure 9).

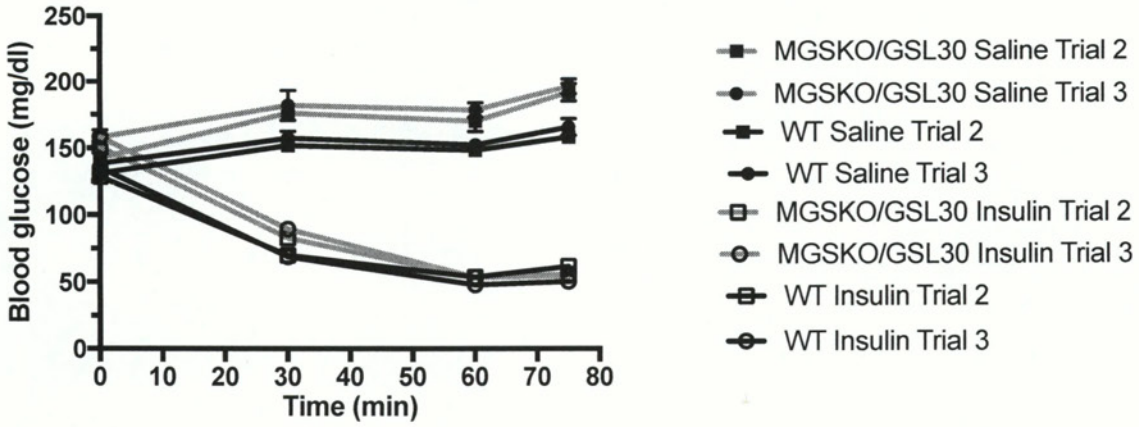

Figure (9): Blood glucose levels were lowered to hypoglycemic levels (40-60mg/dl) in insulin treated groups. Blood glucose levels were measured prior to injection, 30 minutes following injection, 60 minutes following injection, and following rotarod assessment in both WT and *GYS1* $^{-/-/tg}$mice. N=38-40 for each group. Significance was measured using a Newman-Keuls multiple comparison test within a one-way ANOVA.

Blood glucose levels during trial 4 (day 8) – saline treated mice:

Blood glucose was monitored in both WT and *GYS1* $^{-/-/tg}$ mice previously treated with vehicle during trial 4 prior to and following rotarod assessment. There was no difference in blood glucose levels between WT and *GYS1* $^{-/-/tg}$ blood glucose levels prior to rotarod assessment (figure 10). On average WT and *GYS1* $^{-/-/tg}$ mice experienced blood glucose levels of 198mg/dl and 211mg/dl (respectively) prior to rotarod assessment (figure 10). *GYS1* $^{-/-/tg}$ mice had significantly higher blood glucose levels following rotarod assessment when compared to WT blood glucose levels following assessment ($p<0.001$) (figure 10). There was no difference in blood glucose levels following rotarod assessmentwhen compared to post assessment blood glucose in both WT and *GYS1* $^{-/-/tg}$ mice (figure 10). WT and *GYS1* $^{-/-/tg}$ experience average blood glucose levels of 221mg/dl and 246mg/dl (respectively) following rotarod assessment (figure 10).

Blood glucose levels during trial 4 (day 8) – insulin treated mice:

Blood glucose was monitored in both WT and *GYS1* $^{-/-/tg}$ mice previously treated with insulin during trial 4 prior to and following rotarod assessment. There was no difference in blood glucose levels between WT and *GYS1* $^{-/-/tg}$ blood glucose levels prior to rotarod assessment (figure 10). On average WT and *GYS1* $^{-/-/tg}$ mice experienced blood glucose levels of 201mg/dl and 220mg/dl (respectively) following rotarod assessment (figure 10). *GYS1* $^{-/-/tg}$ mice had significantly higher blood glucose levels following rotarod assessment when compared to WT blood glucose levels following assessment ($p<0.001$) (figure 10). On average *GYS1* $^{-/-/tg}$ mice experienced higher blood glucose levels following rotarod assessment when compared to WT mice ($p<0.01$) (figure 10). WT and *GYS1* $^{-/-/tg}$ mice experience average blood glucose levels of 233mg/dl and 249mg/dl (respectively) following rotarod assessment (figure 10).

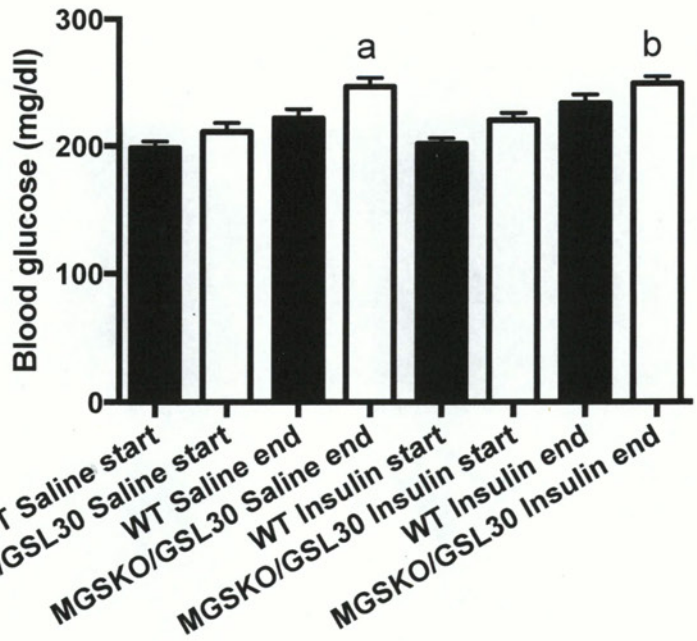

Figure (10): Blood glucose levels were higher in GYS1$^{-/-/tg}$ than WT mice following rotarod assessment. Blood glucose levels were monitored prior to rotarod exposure and immediately following assessment in both WT and GYS1$^{-/-/tg}$ mice. N=38-40 for each group. Significance was measured using a Newman-Keuls multiple comparison test within a one-way ANOVA.

Blood lactate levels

Blood lactate was measured in both WT and *GYS1* $^{-/-/tg}$ prior to and following rotarod assessment in order to investigate compensatory mechanisms during motor learning.

Blood lactate levels pre-rotarod assessment

There was no significant different during trials 1 and 4 when comparing WT and *GYS1* $^{-/-/tg}$ blood lactate prior to rotarod assessment (figure 11). During trial 2, there was no difference saline treated WT and *GYS1* $^{-/-/tg}$ prior to rotarod assessment. However, during trial 2 insulin treated *GYS1* $^{-/-/tg}$ mice experienced higher blood lactate levels when

compared to WT mice ($p<0.0001$) (figure 11). During trial 3, *GYS1* $^{-/-/tg}$ mice experienced significantly higher blood lactate levels than both insulin and saline treated WT mice ($p<0.05$) (figure 11).

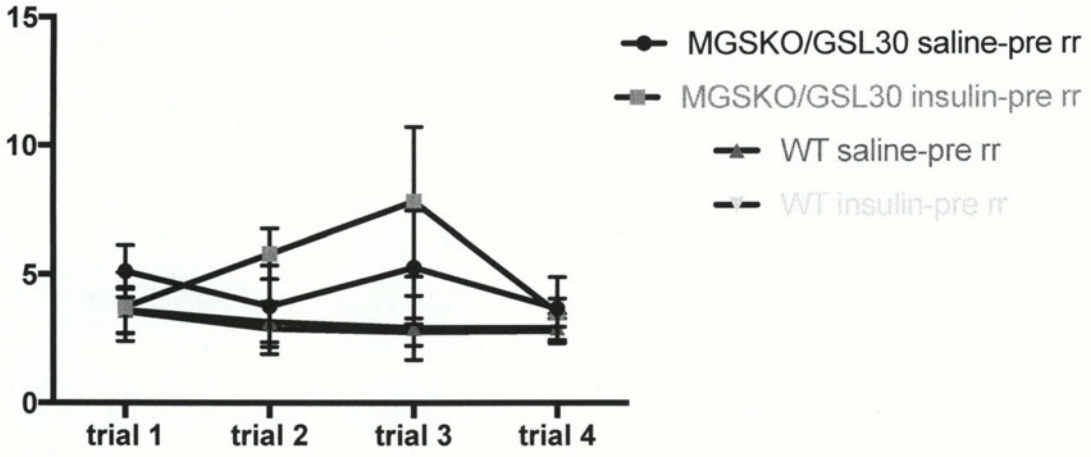

Figure (11): During trials 2 and 3, prior to rotarod exposure, blood lactate levels were higher in GYS1$^{-/-/tg}$ than WT mice. Blood lactate was measured prior to rotarod assessment across all four trials in both WT (n=5) and GYS1$^{-/-/tg}$ (n=5). A non-parametric t-test was used to evaluate signifigance in blood lactate levels between the two genotypes.

Blood lactate levels post-rotarod assessment

Consistent throughout all four trials, blood lactate levels were higher in *GYS1* $^{-/-/tg}$ mice than both insulin and saline treated WT mice following rotarod assessment. ($p<0.05$) (figure 12).

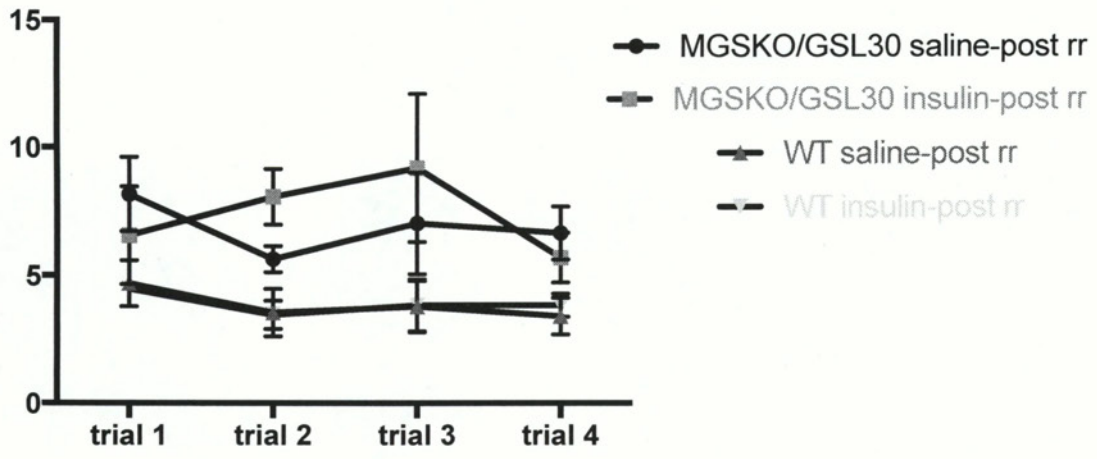

Figure (12): Consistent throughout all four trials, following rotarod assessment, **blood lactate levels were higher in GYS1$^{-/-/tg}$ than WT mice.** Blood lactate was measured immediately following rotarod assessment across all four trials in both WT (n=5) and GYS1$^{-/-/tg}$ (n=5). A non-parametric t-test was used to evaluate signifigance in blood lactate levels between the two genotypes.

Blood lactate levels pre-rotarod vs. post-rotarod

Consistent throughout all four trials, blood lactate levels were unchanged between pre and post rotarod in WT insulin treated mice, WT saline treated mice, and *GYS1* $^{-/-/tg}$ saline treated mice (figure 13). During trials 1, 3, and 4 blood lactate levels were significantly higher in insulin treated *GYS1* $^{-/-/tg}$ mice post rotarod assessment when compared to blood glucose levels prior to rotarod ($p<0.05$) (figure 13). Pre and post rotarod blood lactate levels showed no significant difference in insulin treated *GYS1* $^{-/-/tg}$ mice ($p=0.1$)(figure 13). There is a nearly significant increase ($p=0.08$) in mean lactate levels following rotarod exercise across all four trials (table 1)

Average increase in blood lactate levels (mg/dl) following rotarod exercise				
	Trial 1	Trial 2	Trial 3	Trial 4
WT-saline	0.91	0.4	0.85	0.48
WT-insulin	1.08	0.58	0.85	1.03
GYS1 -/-/tg -saline	3.05	1.88	1.75	2.98
GYS1 -/-/tg -insulin	2.81	2.28	1.38	2.2

Table 1: Blood lactate level increase following exposure to rotarod was significantly higher in GYS1$^{-/-/tg}$ mice. Blood lactate was measure prior to rotarod exercise and immediately following exercise across all four trials in both WT (n=5) and GYS1$^{-/-/tg}$ (n=5). Significance was measured using a Newman-Keuls multiple comparison test within a one-way ANOVA.

Latency

Latency to fall during level 7 (3-30RPM acceleration) rotarod

During trial 1 (day 1) mice underwent the rotarod while in the fed state and no difference was observed in latency between WT and *GYS1* $^{-/-/tg}$ mice (figure 14). Mice were fasted prior to trial 2 (day 2) and received either an insulin or vehicle injection 60 minutes prior to rotarod exposure. No difference in latency was observed between saline treated WT and *GYS1* $^{-/-/tg}$ mice, nor was there difference in latency to fall between insulin treated WT and *GYS1* $^{-/-/tg}$ mice (figure 14). *GYS1* $^{-/-/tg}$ mice treated with insulin had significantly shorter latency when compared to saline treated *GYS1* $^{-/-/tg}$ mice across trials 2,3, and 4 ($p<0.0001$) (figure 14). Both WT and *GYS1* $^{-/-/tg}$ mice insulin treated mice experienced significantly lower latency to fall times during trials 2 and 3 when compared to saline treated WT mice ($p<0.0001$) (figure 14). During trial 4, these insulin and saline

treated WT mice, showed no difference in latency (p=0.1433) (figure 14). Insulin treated *GYS1* $^{-/-/tg}$ mice experienced a shorter latency during trial 4 when compared to WT mice treated with insulin (p=0.0174) (figure 14). There was no differences observed in latency to fall times during trial 4 between saline treated *GYS1* $^{-/-/tg}$ mice and saline treated WT mice.

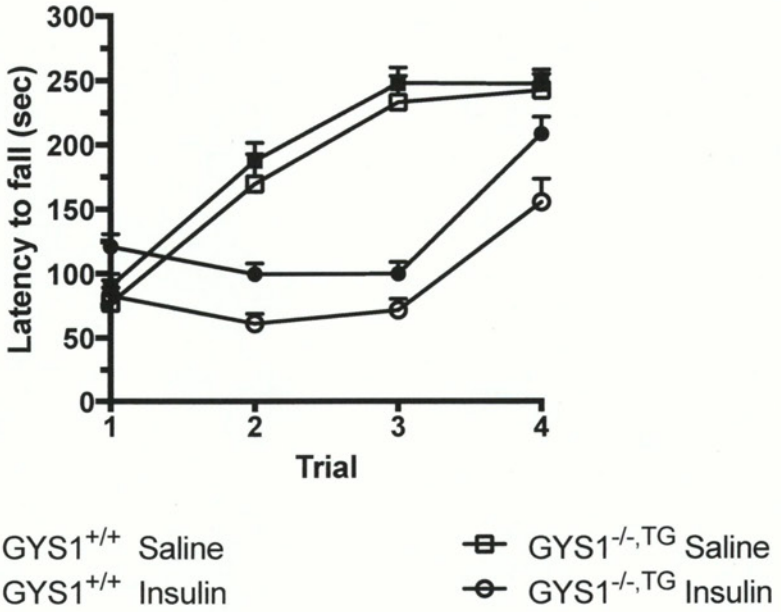

Figure (14): Saline treated mice consistently had longer latency across all four trials on level 7 (3-30RPM) of the rotarod; insulin treated WT mice had longer latency in trial 4 when compared to GYS1$^{-/-/tg}$. Both WT and GYS1$^{-/-/tg}$ mice underwent four trials over a course of eight days on the single rotation rotarod treadmill, n was 18-19 for each group. Mice were treated with either insulin to induce hypoglycemia or saline in order to analyze the effect of hypoglycemia on motor memory formation. Significance was measured using a Newman-Keuls multiple comparison test within a one-way ANOVA.

Latency to fall during level 3 (28RPM) rotarod

During trial 1 (day 1) mice underwent the rotarod assessment while in the fed state and no difference was observed in latency between WT and *GYS1* $^{-/-/tg}$ mice (figure 15). Saline treated WT showed no significant difference in latency when compared to

saline treated *GYS1* $^{-/-/tg}$ mice in all trials (1-4). During both trials 2 and 3, there was no difference in latency to fall times between saline treated WT mice and saline treated *GYS1* $^{-/-/tg}$ mice, nor was there difference observed between insulin treated WT mice and insulin treated *GYS1* $^{-/-/tg}$ mice (figure 15). Insulin treated WT and *GYS1* $^{-/-/tg}$ mice showed significantly lowered latency compared to saline treated WT and *GYS1* $^{-/-/tg}$ mice, respectively, during both trials 2 and 3 ($p<0.0001$) (figure 15). Insulin treated *GYS1* $^{-/-/tg}$ mice had significantly lower latency than both insulin treated WT mice and saline treated *GYS1* $^{-/-/tg}$ mice during trial 4 ($p<0.0001$) (figure 15). Latency to fall times weren't different in insulin treated WT mice compared to saline treated WT mice during trial 4 ($p=0.1742$) (figure 15).

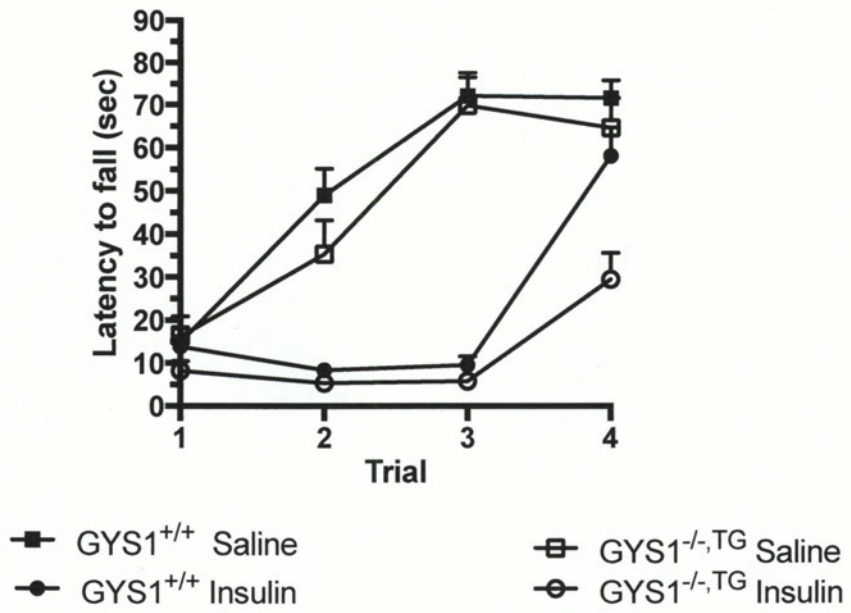

Figure (15): Saline treated mice consistently had longer latency across all four trials on level 3 (28RPM) of the rotarod. There was no difference between WT saline, WT insulin, and GYS1$^{-/-/tg}$ saline treated mice during trial 4, insulin treated GYS1$^{-/-/tg}$ mice had significantly lower latency during trial 4 than the other 3 treatment groups. Both WT and GYS1$^{-/-/tg}$ mice underwent four trials over a course of eight days on the single rotation rotarod treadmill, n was 18-19 for each group. Mice were treated with either insulin to induce hypoglycemia or saline in order to analyze the effect of hypoglycemia on motor memory formation. Significance was measured using a Newman-Keuls multiple comparison test within a one-way ANOVA.

Western Blotting

Hippocampal, cortex, and cerebellum tissue samples were subjected to western blot analysis in order to quantify memory protein KIF17 expression following treatment to rotarod. Consistent with previous studies, KIF17 had a molecular weight of ~170KD, but no definitive conclusions could be made concerning differential expression in differing brain regions or differential memory formation following hypoglycemia.

Discussion:

This study was centered on the hypothesis that glycogen acts as a neuroprotective agent during times of hypoglycemia due to its ability to yield alternative energy sources to glucose, such as lactate and glutamate, following glycogenolysis. Glycogen stored in the astrocytes has been proposed to serve as a source of glucose substitutes which can be shuttled into neurons, which can be utilized to produce the energy needed for neuronal activity during times of hypoglycemia. The role of glycogen was investigated in the formation and recollection of motor memory in differing areas of the brain, including the hippocampus and cerebellum.

Motor memory formation in the fed state

In order to evaluate glycogen's role in memory formation during times of euglycemia, both WT and $GYS1^{-/-/tg}$ were subjected to rotarod while in the fed state. There was no difference in improvement over the course of the four trials between the two genotypes (figure 6), suggesting both genotypes are equally capable of forming memory involving motor skills. These results support the conclusion that glycogen acts primarily as an emergency energy reserve (Suzuki 2011), rather than the primary fuel source needed to support neuronal activity during memory formation. This is in contrast to studies that indicate an important role for brain glycogen in associative memory (Newman, Suzuki, Duran). Thus it appears that different types of learning may have different requirements for brain glycogen utilization. Due to the ability of $GYS1^{-/-/tg}$ mice to learn at an equal rate to WT mice during euglycemic conditions, it is hypothesized that differences in learning between the two genotypes, occurring in the hypoglycemic state,

is due to the utilization of glycogen reservoirs within the astrocytes of the brain. Therefore, we expect the mice lacking brain glycogen, $GYS1^{-/-/tg}$, will have decreased levels of motor memory formation during times of hypoglycemia when compared to WT mice.

Motor memory formation during times of hypoglycemia

Insulin induced hypoglycemia

In order to investigate glycogen's possible role as a neuroprotective agent against memory loss, both WT and $GYS1^{-/-/tg}$ mice were exposed to rotarod treadmill while hypoglycemic or euglycemic. The mice underwent four trials over a course of eight days on the rotarod. Blood glucose was taken prior to and immediately following rotarod exercise during trials 1 and 4, and taken prior to injection, 30 minutes following injection, 60 minutes following injection, and immediately following rotarod during trials 2 and 3. Blood glucose was altered using insulin injections during trials 2 and 3 and consistently brought down to a range between 40-60mg/dl (figure 9). These levels are indicative of hypoglycemia, signifying that 0.6U/kg for WT mice and 1.0 U/kg for $GYS1^{-/-/tg}$ mice were sufficient levels of insulin to induce hypoglycemia within an hour of the time of injection.

On average there was no difference in blood glucose levels in the two genotypes following trial 1 of the rotarod (figure 8). However, both genotypes experienced significantly higher blood glucose levels following rotarod when compared to the rested state, demonstrating the increased need for energy provided by glucose during times of

stress. This stress can be accounted for by both transportation into the experimental room from a separate room as well as the exposure to the rotarod machine in which the mice are learning new motor skills and having to exert energy to do so. Following rotarod assessment on the final trial, $GYS1^{-/-/tg}$ mice had significantly higher blood glucose levels when compared to WT mice (figure 10). Because experimental procedures were consistent between both genotypes, these results insinuate that the increase in blood glucose following rotarod during trial 4 is due to the genetic alteration that results in overaccumulation of muscle glycogen in $GYS1^{-/-/tg}$ mice.

Hypoglycemic motor memory during level 7 (accelerating 3-30RPM) rotarod exposure

During trial 1 of the experiment, there was no difference in latency between the two genotypes. The similarity in latency indicates that there is no difference between genotypes in balance and coordination (figure 13). The night prior to trials 2 and 3, mice were fasted in order to ensure they would reach hypoglycemic levels when injected with insulin. Consistent in both trials 2 and 3, saline treated $GYS1^{-/-/tg}$ mice had similar latency as WT mice, while insulin treated $GYS1^{-/-/tg}$ and WT mice both had shorter latency on the rotarod (figure 13). The lack of difference in latency between saline treated WT and $GYS1^{-/-/tg}$ mice suggests equal learning capacity when it comes to motor skills regardless of brain glycogen levels. The significantly shorter latency in insulin treated groups when compared to saline treated groups indicates that motor skills are impaired during times of hypoglycemia. These results are consistent with clinical studies suggesting that learning in humans is compromised while blood glucose levels are low (Sommerfield 2003).

Mice were in a euglycemic state when exposed to the rotarod machine during trial 4 to evaluate the level of learning that occurs while in the hypoglycemic state. WT mice that underwent the rotarod experiment while hypoglycemic during trials 2 and 3 had no difference in latency during trial 4 when compared to WT and $GYS1^{-/-/tg}$ mice treated with saline during trials 2 and 3 (figure 13). $GYS1^{-/-/tg}$ mice induced with hypoglycemia during trials 2 and 3 experienced significantly shorter latency when compared to saline treated WT and $GYS1^{-/-/tg}$ mice as well as insulin treated WT mice. These results suggest a decreased capacity to learn new motor skills while in the hypoglycemic state without glycogen to serve as an emergency energy reservoir. There was no difference in latency between WT mice previously exposed to insulin-induced hypoglycemia and WT mice treated with saline. This evidence supports our hypothesis that glycogen is able to act as a neuroprotectant against memory loss and is able to provide an alternative energy source to glucose for neuronal activity. $GYS1^{-/-/tg}$ mice lacking brain glycogen were unable to recover memory formed while in the hypoglycemic state, suggesting a lack of fuel needed to support the neuronal activity required for memory formation. Without a glycogen reservoir to supply alternative energy sources to the neurons, mice were unable to form memory of the motor skills learned while in insulin-induced hypoglycemia.

Hypoglycemic motor memory during level 3 (consistent 28RPM) rotarod exposure

In order to study the fast state of motor memory, mice were subjected to the rotarod at level 3, spinning at a constant 28RPM, following exposure to level 7 on the rotarod. This increased speed prevents the mice from "easing" into learning the motor skills required for the rotarod treadmill. The results seen concerning improvement in

rotarod performance across the four trials is comparable to the results of level 7 learning (figure 13). During trial 1, both WT and GYS1$^{-/-/tg}$ mice preformed at a comparable level, resulting in similar latency to fall times. This result further supports the notion that motor skills can be developed at a parallel rate in mice containing brain glycogen and those null of brain glycogen (figure 14). During both trials 2 and 3, insulin treated WT and GYS1$^{-/-/tg}$ mice had significantly reduced latency to fall times when compared to mice treated with saline, once again indicating motor skills are impaired during times of hypoglycemia (figure 14). During trials 2 and 3, there was no difference in latency to fall times when comparing hypoglycemic WT mice to hypoglycemic GYS1$^{-/-/tg}$ mice. Both genotypes of mice were equally impaired while under insulin-induced hypoglycemia, suggesting glycogen doesn't prevent motor skill impairment during hypoglycemic periods.

During trial 4 of the experiment, WT mice that experienced insulin-induced hypoglycemia during trials 2 and 3, had comparable latency as saline treated WT and GYS1$^{-/-/tg}$ mice. The similar latency suggests that during the fast state of motor memory formation, glycogen plays a neuroprotective role against memory loss during periods of hypoglycemia. Unlike WT mice, GYS1$^{-/-/tg}$ mice had significantly shorter latency to fall times than saline treated mice, indicating they were unable to recover memory formed during trials 2 and 3 when they were hypoglycemic. At both level 7 and level 3, mice lacking brain glycogen had significantly impaired latency times during trial 4. These results suggests a lack of memory formation, supporting the hypothesis that glycogen acts as a neuroprotective agent against memory loss and effectively assists in memory formation during insulin-induced hypoglycemia.

Lactate measurements

Although the results support the hypothesis of glycogen's role as a neuroprotectant, exactly how this polymer assists in memory formation and recollection is still not clear. We hypothesized that substrates resulting from glycogenolysis, specifically lactate can be shuttled from astrocytes into neurons and used as an alternative energy source to glucose. Evidence supporting this hypothesis show that inhibiting lactate transport between brain cells by blocking the MCT induction disrupts memory formation (Suzuki 2011). In order to test this hypothesis, we measured blood lactate prior to and immediately following rotarod exercise. Consistent throughout all four trials, blood lactate levels were significantly higher in $GYS1^{-/-/tg}$ mice than in WT mice following exercise on the rotarod (figure 11). This is consistent with the increased levels of muscle glycogen within $GYS1^{-/-/tg}$ mice being degraded to lactate during muscle contractions while walking on the rotarod. Although WT mice had lower levels of blood lactate than $GYS1^{-/-/tg}$ mice, their blood lactate levels still increased significantly following rotarod when compared to lactate levels prior to exposure (table 1). The result that lactate is increasing in the blood following rotarod, could result in increased delivery of lactate from the blood to the brain, thus providing neurons with a substrate that can be converted into pyruvate and utilized in the TCA cycle to generate ATP required for memory formation. These results support the hypothesis that lactate may be utilized in the brain as an alternate to glucose during times of hypoglycemia and is consistent with papers indicating lactate is required for memory formation (Suzuki 2011).

Western Blot Analysis:

In order to further investigate the role of brain glycogen as a neuroprotectant against memory loss, a western blot analysis was utilized to track protein expression in different regions of the brain. We hypothesized that mice lacking brain glycogen would show decreased levels of memory protein expression due to their lack of an alternative energy source to glucose that glycogen provides during hypoglycemia. The cerebellum, hippocampus, and cortex (figure 4) were all dissected approximately 24 hours following trial 4 of the rotarod experiment. The brains were than homogenized and blotted to analyze the expression of the motor protein KIF17. Although expression of the protein has yet to shown to be directly affected by motor memory, it has been shown to be interconnected with other proteins that change in expression level following motor memory formation (Roberson 2008). The western blot resulted in bands at ~170kD, indicating the protein is expressed in the three regions of the brain. Expression of the protein was shown to be comparable across treatment groups and genotypes, however no conclusive evidence can be drawn from this as it was only duplicated a single time. More western blots analysis is needed to provide further evidence of KIF17's expression during memory formation.

Conclusion:

Using the rotarod treadmill, we have shown that brain glycogen is not a required component for memory formation while in the euglycemic state, demonstrated by the equal performance of both WT and $GYS1^{-/-/tg}$ mice across all four trials on the rotarod. Brain glycogen was shown to play an important role in memory formation and recollection during times of hypoglycemia as WT mice exposed to insulin induced

hypoglycemia preformed at a significantly higher rate during the last trial on the rotarod when compared to GYS1$^{-/-/tg}$ mice previously exposed to insulin-induced hypoglycemia. These results suggest that WT mice have access to glycogen resivours within astrocytes during hypoglycemic periods and this glycogen improved motor memory formation when blood glucose is unavailable. The results from this experiment also suggest motor skills are significantly impaired during times of hypoglycemia, as seen by the significantly shorter latency in insulin treated groups as compared to saline treated groups. Analyzing the expression of KIF17 in mice of differing brain glycogen levels provided no conclusive evidence into glycogen's role as a neuroprotectant.

Using blood glucose measurements, this experiment indicated the optimal dosage of insulin to bring WT and GYS1$^{-/-/tg}$ mice down to hypoglycemic levels. It also indicated the overexpression of glycogen synthase GYS1$^{-/-/tg}$ mice causes significantly higher blood glucose levels following times of stress when compared to WT mice. Lactate was also raised at a substantially higher rate in mice overexpressing muscle glycogen as evidenced by increased levels of blood lactate in GYS1$^{-/-/tg}$ mice compared to WT mice. This lactate could act as a compensatory mechanism providing the energy production needed to support neuronal cell activity.

Future directions:

In order to better understand the role brain glycogen plays in motor memory formation, future rotarod experiments will be conducted using different models of mice. Due to the genetic makeup of GYS1$^{-/-/tg}$ mice that leads to overaccumulation of muscle glycogen, the mice could be adapting to their physiological excess of muscle glycogen by

shuttling glycogenolytic substrates into the brain to be used as alternative energy source in neurons. A mouse model completely null of brain glycogen, but containing normal levels of muscle glycogen would be beneficial because similarity to WT mice concerning levels of glycogen outside of the central nervous system.

Aside from a mouse model null of brain glycogen that contains normal glycogen levels outside of the CNS, other memory proteins could be analyzed using a western blot to evaluate glycogen's role in memory formation. Proteins such as CREB, pCREB, and Arc have been demonstrated to be involved in motor memory formation, thus differing expression levels in a western blot analysis would provide insight into glycogen's role in memory formation.

Works cited:

1. 2006. American Diabetes Association. Diabetes Care, suppl. American Diabetes Association: Clinical Practice Alexandria 29:1

2. 2012. American Diabetes Association: http://www.diabetes.org/diabetes-basics/statistics/?referrer=https://www.google.com/

3. Amaral, Ana L. 2012. Effects of Hypoglycemia on Neuronal Metabolism in the Adult Brain: Role of Alternative Substrates to Glucose, Journal of Metabolic Inherited Diseases 30: 1-14

4. Bolo B R, et al. 2011. Brain Activiation During Working Memory Is Altered in Type 1 Diabetes During Hypoglycemia, Diabetes 1-9

5. Chagniel L, Bergeron Y, Bereau G, Massicotte G, and Cyr M. 2013. Regulation of Tyrosine Phosphatase STEP61 by Protein Kinase A during Motor Skill Learning in Mice, PLoS ONE 9(1): e86988

6. De Zeeux C I and Brink M M. 2015. Motor Learning and the Cerebellum, Cold Spring Harbor Perspectives in Biology 1-20

7. Doyon J, Bellec P, Amsel R, Penhune V, Monchi O, et al. (2009) Contributions of the basal ganglia and functionally related brain structures to motor learning. Behav Brain Res 199: 61–75

8. Herzog I R, Sherwin R S, & Rotham D L. 2011. Insulin-Induced Hypoglycemia and Its Effect on the Brain, Diabetes 60(7): 1856-1858

9. Manchester J, et al. 1996. Increased Glycogen Accumulation in Transgenic Mice Overexpressing Glycogen Synthase in Skeletal Muscle, Biochemistry 93: 10707-10711

10. Min R, et al. 2014. Arc Regulates Experience-Dependent Persistent Firing Patterns in Frontal Cortex, The Journal of Neuroscience 34(19): 6583-6595

11. Pederson B A, et al. 2004. Abnormal Cardiac Development in the Absence of Heart Glycogen, Molecular and Cellular Biology 24(16): 7179-7187

12. Reijmer Y D, et al. 2010. Cognitive Dysfunction in Patients with Type 2 Diabetes, Diabetes 26: 507-519

13. Roberson R, et al. 2008. Altered Expression of KIF17, a Kinesin Motor Protein Associated with NR2B Trafficking, May Mediate Learning Deficits in a Down Syndrom Mouse Model, Am Journal of Obstet Gynecol 198(3): 313.e1-313.e4

14. Suzuki A, et al. 2011. Astrocyte-Neuron Lactate Transport Is Required for Long-Term Memory Formation, Cell 144(5): 810-823

Office of Research Integrity
Institutional Animal Care and Use Committee
2000 University Avenue
Muncie, Indiana 47306-0155
Phone: 765-285-5070

DATE:	November 25, 2015
TO:	Bart Pederson
FROM:	Ball State University IACUC
RE:	IACUC Protocol #: 135559-24
TITLE:	The role of glycogen in development, metabolism, and disease
SUBMISSION TYPE:	Other
ACTION:	**APPROVED**
DECISION DATE:	November 25, 2015
EXPIRATION DATE:	November 24, 2018
REVIEW TYPE:	Full Committee Review

The Institutional Care and Use Committee (IACUC) recently reviewed the above names protocol. Your protocol was **APPROVED**.

Approval period: November 25, 2015 through November 24, 2018

Category:

[X] Laboratory Research
[ ] Teaching
[ ] Non-Laboratory Research
[ ] Breeding Colony

Approved Animal Bio-Safety level (ABSL): [X] ABSL 1 [ ] ABSL 2 [ ] No Changes

Bio safety committee (IBC) approval # *[enter if applicable]*

Editorial Notes:

NONE

It is the principal investigator or faculty advisor's responsibility to ensure that all approved research protocols are followed and are in accordance with (when applicable):

PHS Policy on Humane Care and Use of Laboratory Animals;

Guide for the Care and Use of Laboratory Animals;

AVMA Guidelines on Euthanasia; and

All applicable biosafety requirements

As a reminder, it is the responsibility of the principal investigator and/or faculty advisor to inform the IACUC:

- When the project is complete or discontinued (Final Report/Study Closure),
- Report annual updates on the
- If the project is to be continued beyond the approved end date (3-Year Renewal Application)
- If the project is to be modified (Modification/Amendment Form)
- If the project encounters problems (Adverse Events Form)

Please report any of the above situations to the IACUC through IRBNet. Please do so through your currently approved protocol number. Be sure to allow sufficient time for review and approval of requests. If you have any questions regarding this request, please contact Jennifer Weaver at 765-285-5034 or jmweaver@bsu.edu.

Heather A. Burns, PhD, Professor, Chair
Institutional Animal Care & Use Committee

Christopher Mangelli, JD, MS, MEd, CIP, Director
Office of Research Integrity